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14. ABSTRACT

We aim to investigate a Layer-by-Layer (LBL) bioprinting process using stem cells for retinal tissue regeneration. The LBL nature of the bioprinting process matches nicely with the native, multilaminar anatomy of the retina. This research will bioprint retinal stem cells (RSCs) and appropriate growth factors (GF) encapsulated in a biomaterial (e.g. hyaluronic acid, HA). We will investigate the mechanical, chemical and biological properties of the hydrogel materials. We will study the cell viability and analyze the biological functions of the 3D printed retina tissue.

15. SUBJECT TERMS

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1. Introduction

Here is the final report on the project - "Layer-by-Layer Bioprinting of Stem Cells for Retinal Tissue Regeneration". We have developed hydrogel biomaterials with comparable mechanical properties that are 3D printable. We used human fetal retinal progenitor cells (hfRPC) as the cell resource for retinal tissue differentiation. We have demonstrated that these 3D-printed hydrogel materials are biocompatible for retinal cell growth. The hfRPC can be directed toward a specific cell fate within 3D-printed hydrogel and chemically defined induction medium. Moreover, the hfRPC can be differentiated into photoreceptors within a short period of time

2. Keywords

retinal tissue regeneration, 3D bioprinting, Layer-by-Layer, hydrogel biomaterials.

3. Accomplishments

What were the major goals of the project?

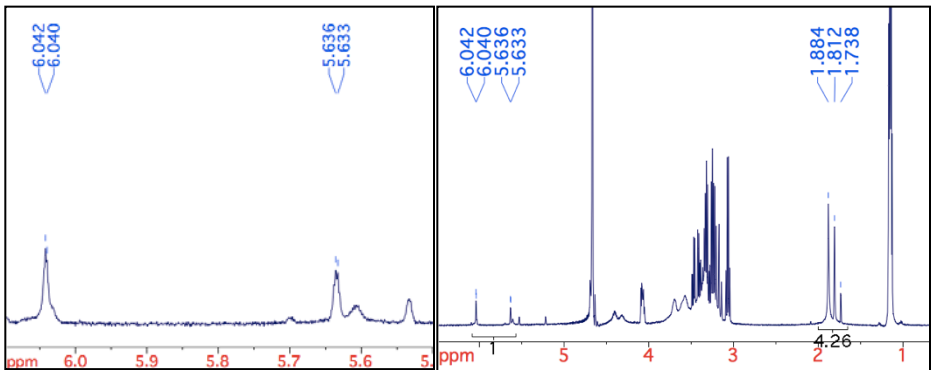
The major goal of this project is to investigate a Layer-by-Layer (LBL) bioprinting process using stem cells for retinal tissue regeneration. Our specific aims are as followings:

Specific Aim 1: Develop and optimize a 3D bioprinting method with encapsulated RSCs,

Specific Aim 2: Layer-by-layer bioprinting of *in vitro* retina PRs/RPE/Bruch's membrane tissues.

a) Synthesis of hyaluronic acid-glycidyl methacrylate hydrogel.

For the synthesis of HA-GM, 200 mg of hyaluronic acid (Lifecore Biomedical, MN) was added into 25ml of 50% acetone solution. The solution was mixed overnight before adding 1.8ml (20-fold molar excess) of triethylamine and glycidyl methacrylate. The reaction took 8 hours before dialysis and lyophilization. The methacrylation was confirmed by ^{1}H-NMR characterization (Joel 500) by the peaks at 5.6 and 6.0 ppm, as shown in **Fig. 1a.** The degree of methacrylation (DM) is determined by integration of the methacrylation group over that of the methyl groups in hyaluronic acid at 1.7, 1.8 and 1.9 ppm, as shown in **Fig. 1b**. For example the DM of the sample in Fig. 1a is 23%. The relation between the initial molar ratio of reagents and the degree of methacrylation of the product is shown in Table 1.



(a) (b)

Figure 1. *The ^{1}H-NMR results of HA-GM showing: a) the methacrylate group; b) the integration of methacrylate group over the methyl groups of hyaluronic acid to calculate the degree of methacrylation.*

***Table 1**. Degree of methacrylation of HA-GM synthesized by different initial stoichiometric ratio*

Initial molar ratio of glycidyl methacrylate to hyaluronic acid	Degree of methacrylation of final HA-GM
20:1	23%
10:1	11%
5:1	3%

b) Mechanical characterization of the hydrogel.

For mechanical testing, the hydrogel sample was prepared by a 3D printing apparatus developed in the lab. A rectangular hydrogel sheet with a dimension of 5mm x 10mm x 1mm was made with the synthesized HA-GM. The elastic modulus of the 3D-printed sheets using HA-GM was determined by tensile tests using a thermomechanical analysis machine (TMA, Perkin Elmer). Briefly, the sample was clamped at two ends. The force applied on the sample started from 3mN with a constant increment of 5mN/min while the elongation of the sample was measured. A stress vs. strain curve is obtained and the elastic modulus is calculated as the slope of the curve. Samples made by HA-GM with methacrylation ratio lower than 23% could not be characterized by this method due to softness. Such softer hydrogels will be measured in the future using atomic force microscopy (AFM). The test result of 2% hydrogel made by HA-GM with 23% methacrylation is shown in **Fig. 2**. The elastic modulus of the hydrogel is about 30±5 kPa. Previous work suggested that the elastic modulus of native retina is about 20 kPa. Compare to other materials used for retina replacement, such as poly (glycerol-sebacate) which has an elastic modulus of 600kPa, HA-GM has closer mechanical property as native retina.

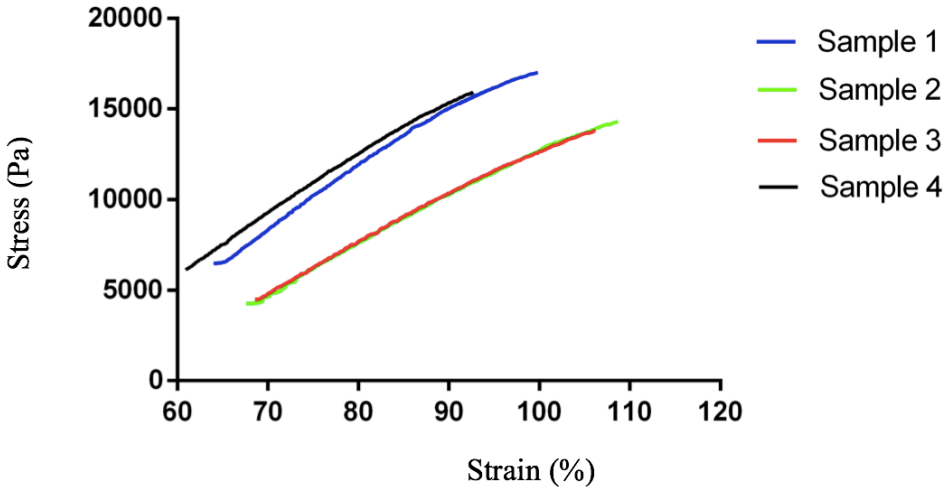


***Figure 2**. Stress vs. strain curve of HA-GM hydrogel with 23% methacrylation ratio*

c) Bioprinter development and optimization.

We have established a strategy to print the HA-GM hydrogel into dome shapes to resemble the native environment for retina development. To improve the cell adhesion within the

hydrogel, we added another printing material, methacrylated gelatin, GelMa, into the HA-GM hydrogel. As the hydrolysis product of collagen, gelatin could facilitate the adhesion of cells due to its chemical structure. We used lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as the free radical generator. LAP has a local absorbance maximum at approximately 375 nm and significant absorbance at 365 nm. The pre-polymer resin included HA-GM, GelMa and LAP. Cell could be mixed into the resin if encapsulation is needed for year 2 tasks.

Fig. 3 shows the 3D printing apparatus developed for this project. Briefly, a UV light

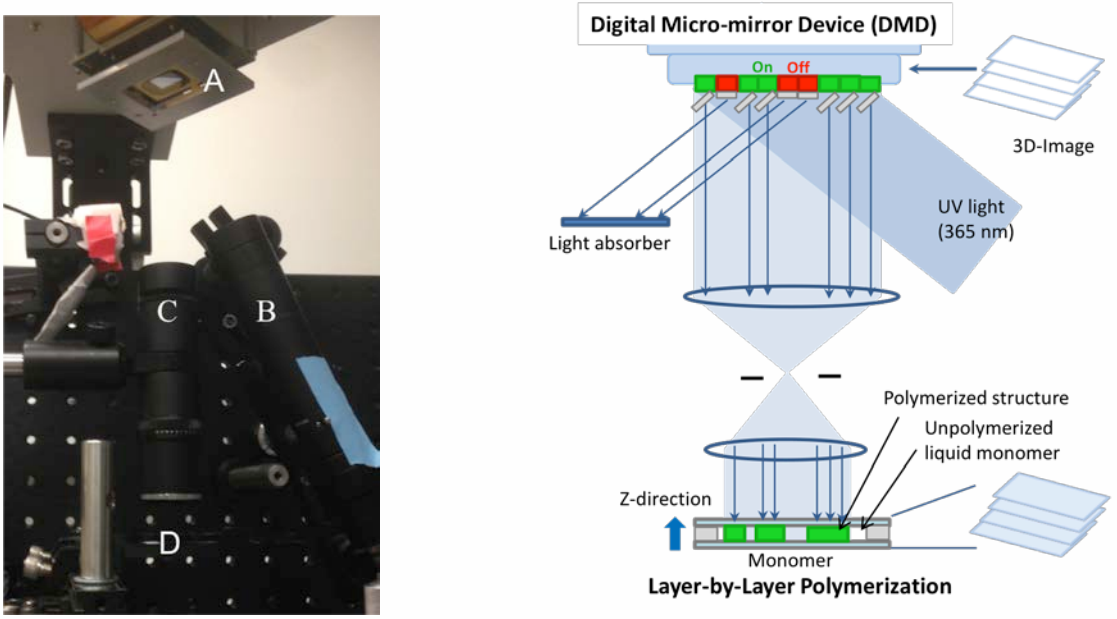


***Figure 3**. Setup of the 3D printer: (Left) The picture of the main part of the 3D bioprinter with labeled components as A: DMD chip; B: lens system to project UV light onto the DMD chip; C: lenses that collimate the light; D: stage that holds the pre-polymer resin. (Right) Schematic showing the components in the 3D bioprinter.*

source (Omnicure S2000, 365nm) is used for photo-polymerization. Patterns are generated by Adobe Photoshop and transferred to a digital-mirror array device (DMD) by in-house software. The DMD chip is used as an optical mask for projecting patterns of light onto the pre-polymer solution for photopolymerization. Once the light passes through the lenses, it is collimated to form a precise image on the pre-polymer solution. The stage could move in all three dimensions, dictated by the design image from the computer. By changing the height of the stage, a 3D polymer scaffold with or without cell can thus be fabricated layer by layer.

d) Layer-by-layer printing using hyaluronic acid - glycidyl methacrylate hydrogel.

The printer enabled us to construct complex structures with the biomaterials. For example, we could print a dome structure with softer material as the core, followed by a harder material as a shell, as shown in **Fig. 4**. Briefly, HA-GM was printed as the first layer in a cylindrical shape with thickness of 125 µm and diameter of 1,500µm. The shell was printed with HA-GM and GelMA mixture. It was printed in the same shape as the first layer with a thickness of 250 µm and diameter of 2,500 µm. With our printer and biomaterial, we could deliver a layer-by-layer printing strategy for retina regeneration *in vitro*.

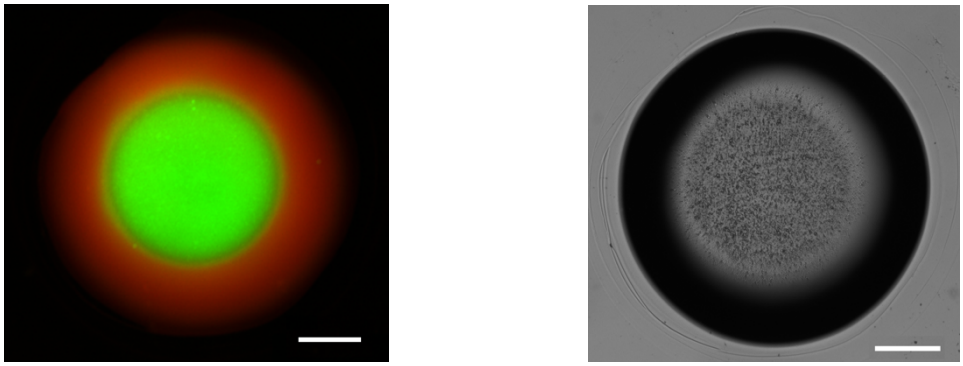


Figure 4*. A dome structure constructed by the 3D printer using biocompatible HA-GM and GelMA hydrogel. Green color comes from fluorescein isothiocyante-dextran in HA-GM as the first layer; red color comes from the tetramethylrhodamine isothiocyanate-dextran in HA-GM and GelMA as the second layer. (Left) fluorescent image, (right) bright-field image. Scale bar = 500 μm*

e) Layer-by-layer printing encapsulating retinal stem cells.

We developed the layer-by-layer (LBL) bioprinting system for the encapsulation of living cells, particularly retinal stem cells (RSCs). The cell-loaded structures were cultured in vitro to evaluate cell viability and investigate the interactions between cells and the microenvironment created by bioprinting.

Briefly, the RSCs were mixed with the monomer solution made of HA-GM and GelMa by gentle pipetting. The cell-loaded solution was then loaded to the stage for bioprinting. A core-shell structure was printed to mimic the curved layer-by-layer fashion of the native retina tissues (**Figure 5**). This design was also expected to introduce a gradient of nutrition and growth factor diffusion from the outside to the inside of the dome structure, which could guide the orientation of the cells to mimic the highly ordered retinal cells including photoreceptors, ganglion cells and bipolar cells. As shown in Figure 5, we were also able to vary the cell density in each layer to investigate the optimal condition for the differentiation of the RSCs into the cells of different layers in the retinal tissue. The cell density in the core part was 30 million per ml and the cell density in the shell was 15 million per ml.

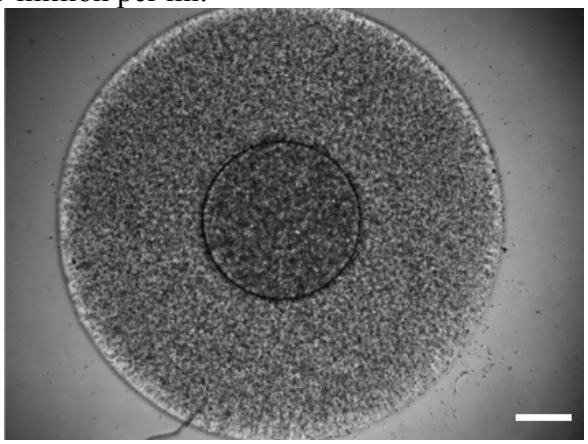


Figure 5. *Bioprinted core-shell structure encapsulated with RSCs. Scale bar: 500 μm.*

After 10 days of *in vitro* culture, we found that some RSCs were spreading in the hydrogel structure along the direction of the radius as well as the interface between the core and the shell (**Figure 6**). Immunofluorescence staining also showed the expression of photoreceptor-specific reporter IRBP-GFP (green) and recoverin (red), indicating photoreceptor induction with the help of differentiation medium (**Figure 7**).

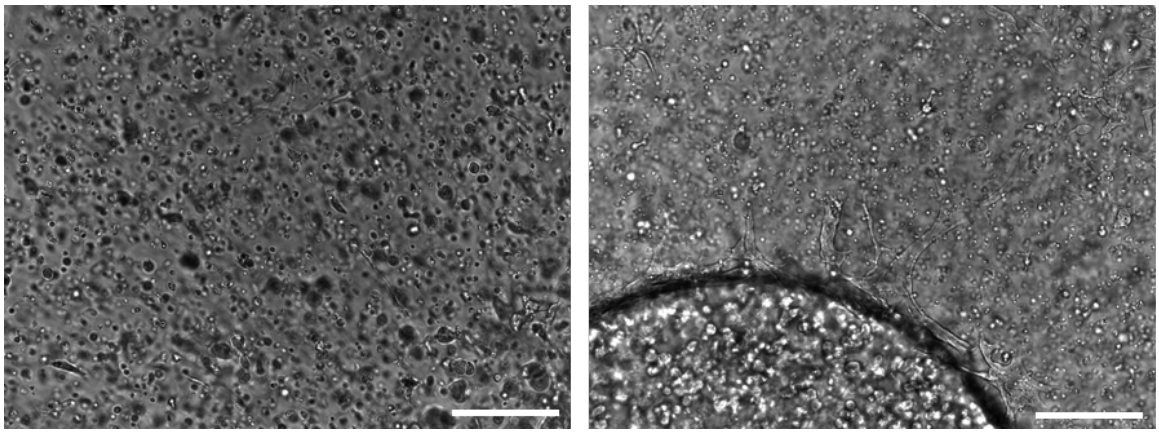


Figure 6. *Encapsulated cells spreading in the shell layer (left) and the interface between the core and the shell at day 10. Scale bar: 250 μm.*

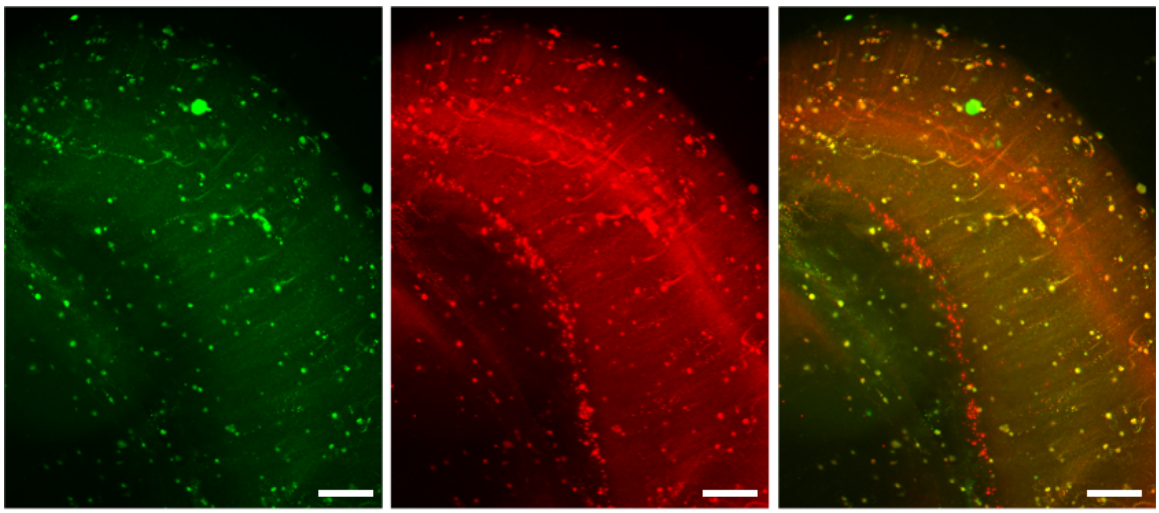


Figure 7. *Characterization of RSC differentiation to photoreceptors at day 10. Green: photoreceptor-specific reporter IRBP-GFP. Red: recoverin. Scale bar: 250 μm.*

To construct a multi-layered structure to facilitate the differentiation of RSCs, a single layered hRPE cells is desired. A uniform spreading of hRPE cells mimics the *in vitro* environment during the retinal formation, thus secreting the necessary growth factors and differentiation signals for the RSCs . We have prepared a single layered hRPE cells by encapsulating the cells into the GelMa solution and printed a thin layer on the cover glass, as shown in **Fig. 8**. At day 2, the hRPE cells were still encapsulated in the gel without spreading. After 4 more days, the cells have detected the stiffer glass surface and migrated to form a single layer.

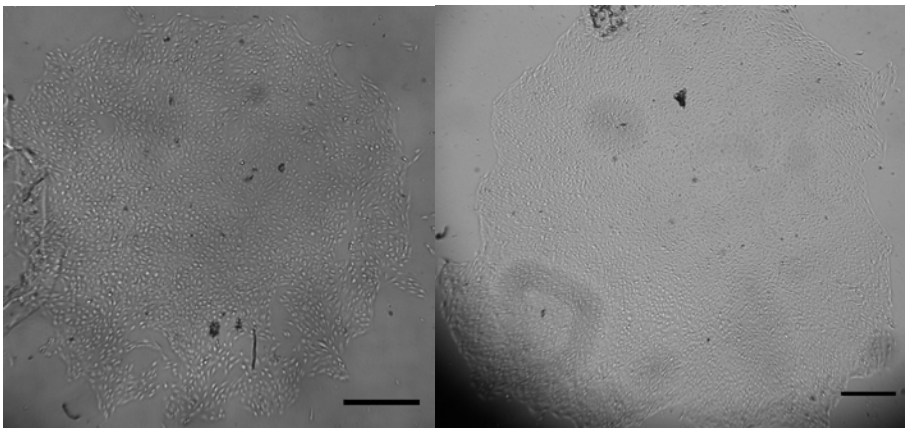


Figure 8. *Formation of single layered hPRE cells: Left: 2 days after printing. Right: 6 days after printing. Scale bar = 200 μm*

f) Differentiation medium development.

To develop an effective culturing medium for maintaining hfRPC and differentiation, we tried several different combinations and eventually set one standard method to culture the hfRPC and a modified medium for the photoreceptor differentiation. The difference between these different methods were measured by the proliferation rates of the hfRPC (Fig. 9),

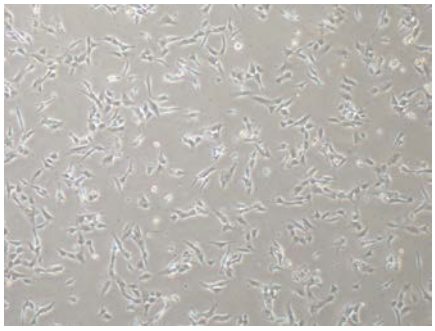


Figure 9. *White field image of under culture hfRPC.*

Different culture mediums were tested for the expansion and differentiation of hfRPCs. The hfRPC expansion medium A (DMEM/ham's F12 nutrient mixture, N2&B27 supplements, 0.05% BSA, 2mM L-GlutaMAX, 0.1mM MEM non-essential amino acids, 0.1mM β-mercaptoethanol, 2μM SB431542, 50nM LDN193189, and 1μM IWP2) was tested. This serum-free medium supplemented by small molecule was capable to expanse hfRPC up to 5 passages with the morphology maintained. Besides, the hfPRC-expansion medium B (Ultraculture serum-free medium with 10ng/ml epidermal growth factor, 20ng/ml beta-fibroblast growth factor, 2mM L-glutamine) was also utilized. The expansion medium B had shown more benefits on hfRPC proliferation and managed to reserve the cell morphology for more than 10 passages. For the photoreceptor generation from hfRPC, the hfRPC differentiation medium (DMEM/ham's F12 nutrient mixture + Neurobasal 1:1, N2&B27 supplements, 0.05% BSA, 2mM L-GlutaMAX, 50nM docosahexaenoic acid, 1μM IWP2+DACT, 100nM purmophine, 100nM retinoic acid and 100μM taurine) was tested.

In order to trace the process of differentiation from hfRPC to photoreceptor cells we labeled the hfRPC with GFP by lentivirus (Fig. 10)

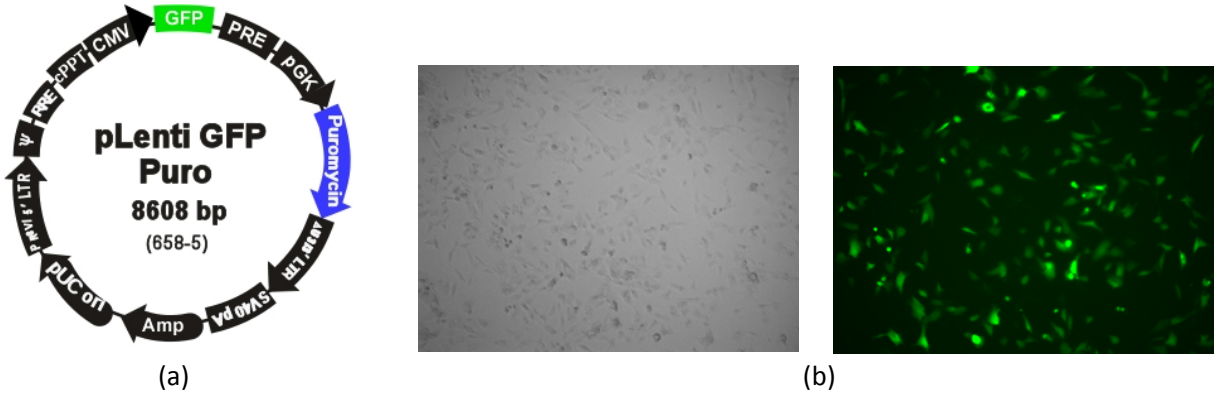


Figure 10. *(a) Vector map of lentivirus-GFP, (b) GFP labeled hfRPC*

g) Bi-layer construct of hRPE and RSC mimicking native retinal development.
We carried out bioprinting of both human hRPEs and hfRPCs in two-layer setup. We then co-differentiated two cells types to induce photoreceptor cells differentiation from hfRPCs. The differentiation was confirmed by both axon formation and immunostaining of recoverin, which is a unique protein marker in photoreceptors.

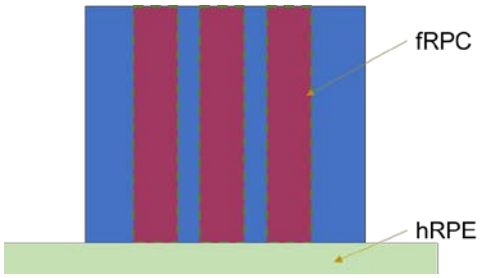


Figure 11*. Two Layer bioprinting of hfRPC and hRPE*

Fig. 11 shows the schematic setup of the two-layer bioprinting of the two cell types. During native retinal development *in vivo,* the two cell types have different extracellular matrices (ECMs). The hRPEs are mostly surrounded by collagen while the hfRPCs reside in a hyaluronic acid environment. To mimic the collagen ECM, the hRPE cells are encapsulated in gelatin methacrylate (GelMa) as the first layer. This layer is about 125 μm thick and cultured three days prior to the second layer so that the hRPE cells will form a single cell sheet. The second layer is the cells encapsulated in both GelMa and hyaluronic acid-glycidyl methacrylate (HA-GM) with thickness of 250 μm. Two layers are adjacent to each other so that the growth factors secreted by hRPEs will assist the differentiation process of the hfRPCs.

To further facilitate the co-differentiation, we added COCO human recombinant protein. The differentiation was examined by immunology staining of recoverin, which is a unique protein in photoreceptors.

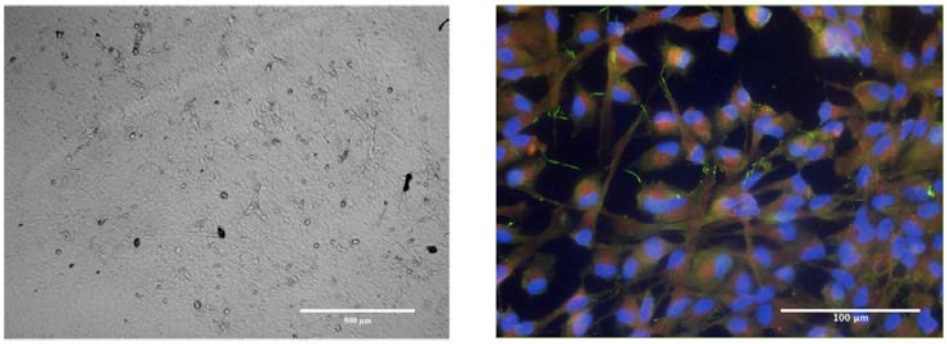
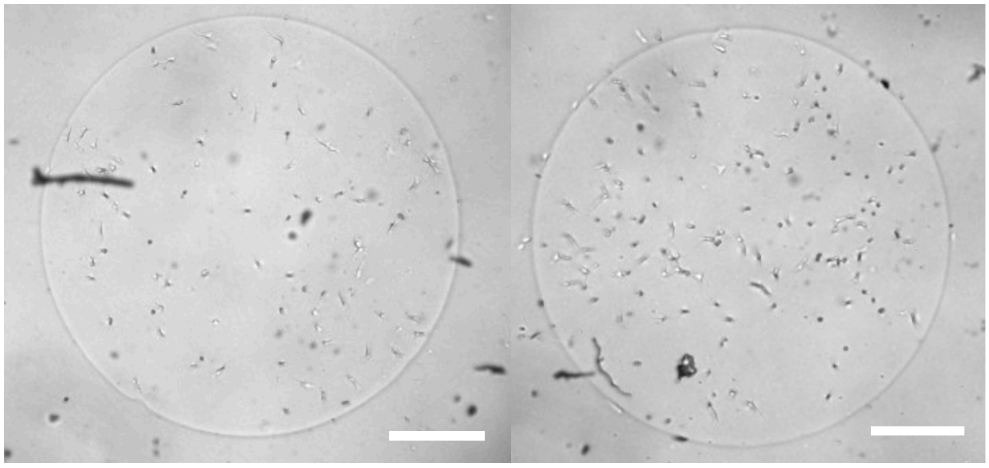


Figure 12*. Co-culturing fRPC and hRPE after 4 days. (a) Bright filed image; (b) Confocal microscopy image showing immunostaining of: recoverin (red), RPE 66 (green) and DAPI (blue)*

In **Fig. 12**, both the bright filed and confocal images of the two-layer co-differentiation after 4 days of culturing are presented. Small fractions of axial formation from RPCs were observed in the bright field image, indicating small degree of differentiation of the fRPCs. The immunostaining of photoreceptor-specific recoverin indicates that some of the fRPCs have differentiated into photoreceptors. The absence of RPE 66 suggests that the cells are from fRPCs. However, large-scale photoreceptors were not present. Additionally, the hRPE layer should withstand the whole differentiation period, which is about 14 days. Currently, the hRPEs will digest the GelMa layer within 5 days. We further improved the setup by using channels to separate the two cell layers. The new design will use much smaller channels and the fRPCs will be positioned within those channels. The hRPE layer will be encapsulated in more biologically stable material at the other sides to provide essential growth factor for the differentiation.

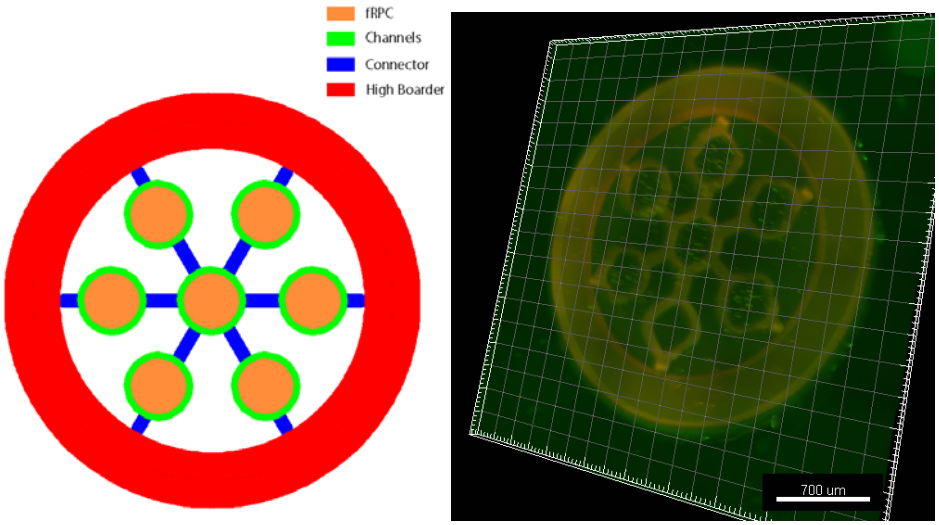
To solve the problem of declining s expansion and differentiation due to hRPEs overgrowth, we proposed to control the hRPEs migration by decreasing the material degradation rate. We investigated the viability of encapsulated hRPEs in less biodegradable material to retain the stability of the hRPE layer. We examined materials made up of 2.5% gelatin-methacrylate (GelMa) with poly (ethylene-glycol) diacrylate (PEGDA) of various molecular weight, as well as concentrations (**Fig. 13)**. Ideally, the hRPEs will expand in an isotropic manner and form a sheet after packing with each other. We observed that the cells, however, stretched anisotropically after 3 days of encapsulation. The cell-cell connections were not present. Our observations indicated that the encapsulation will not achieve the single layer formation by the hRPEs.



(a) (b)

Figure 13. *hRPE cell encapsulation with 2.5% GelMa and PEGDA with molecular weight of (a) 6000 Da and (b) 10000 Da at day 3. Scale bar = 500µm*

To prevent from being marginalized by the growing hRPEs, instead of using a less biodegradable material to delay the hRPEs growth, we chose to take an alternative by separating the two cell types vertically. We designed a bilayer *in vitro* co-culture structure as shown in **Fig. 14a**. The structure will have a channel, held by a non-degradable wall which is linked by a rigid connector to a boarder. Later this structure will be flipped and put on top of a petri dish-cultured hRPE layer to co-differentiate the two cell types together. The boarder will be higher than the centrum components so that there will be a space between the fRPCs and hRPEs to mimic the neural retinal development *in vivo*. We conducted an initial proof-of-concept study on the structure as well as cell viability examination. At day 3, about 70% of the cells encapsulated in the channel are still alive, as shown in **Fig. 14b**.



(a) (b)

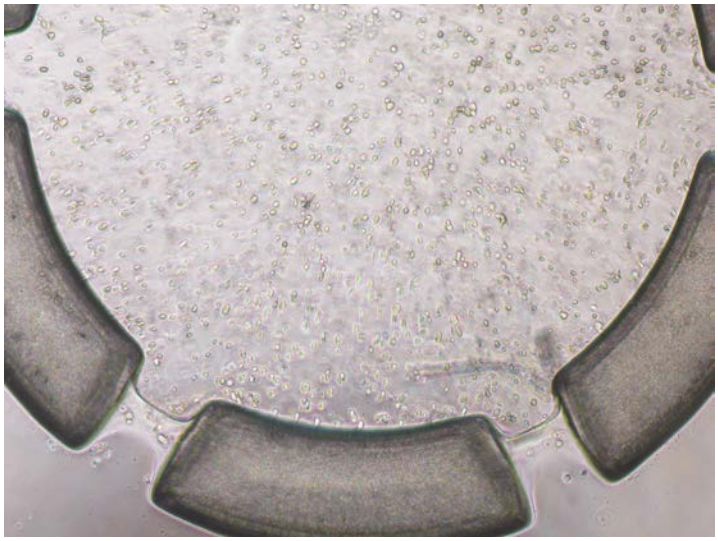


Figure 14. (a) Schematic illustration of cell-encapsulated channel structures; (b) Cell viability test after 3D printing and encapsulation at day 3; (c) white image of hRPEs after 3D-printing day 1.

h) Co-differentiation of hRPE and RSC to induce photoreceptor formation.

We examined the differentiation protocol adapted from previous work. Previously we encountered low viability after printing. We hypothesized both the nutrition diffusion and the differentiation process could cause the cell death. Since only small fraction of the total cell population will be differentiated into photoreceptors, most of the s will undergo apoptosis and eventually die. To investigate how to minimize the cell death, we designed experiment by differentiating single cell type, the fRPCs, and studied the cell viabilities after 7 days. We took both bright field and immunostaining images to exam the differentiation process and the impact on cell survival. We took a white field image, as shown in **Fig. 15**. The arrows indicated apoptotic cells during differentiation

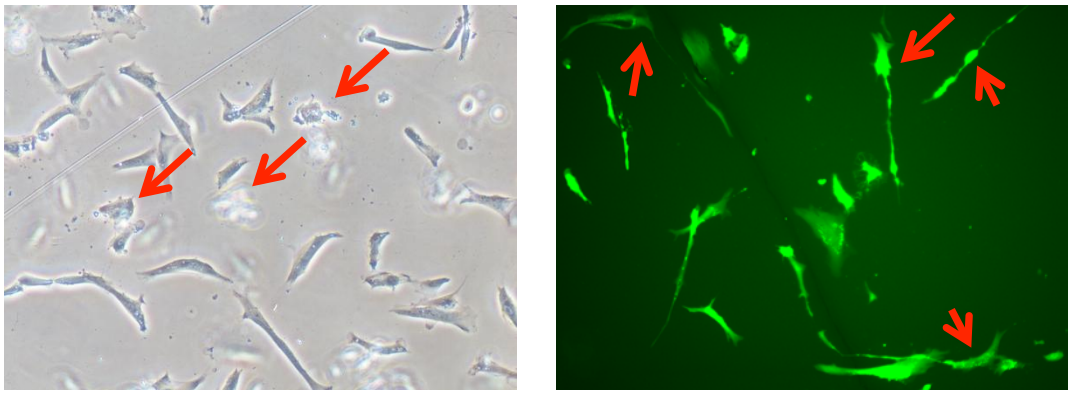


***Figure 15**. Differentiation of fRPCs into photoreceptor-like cells shown in white field image and fluorescent image*

The immunostaining of recoverine, one of the signature proteins in photoreceptors, were further conducted to assess the maturation of fRPCs into photoreceptors, as shown in Fig. 16. Instead of anisotropic original round shape, the fRPCs have grown isotropically and developed interaction with an adjacent cell. This change of morphology indicated that cells have matured into later stage. The immunostaining of recoverine further confirmed that photoreceptors have formed from the fRPCs.

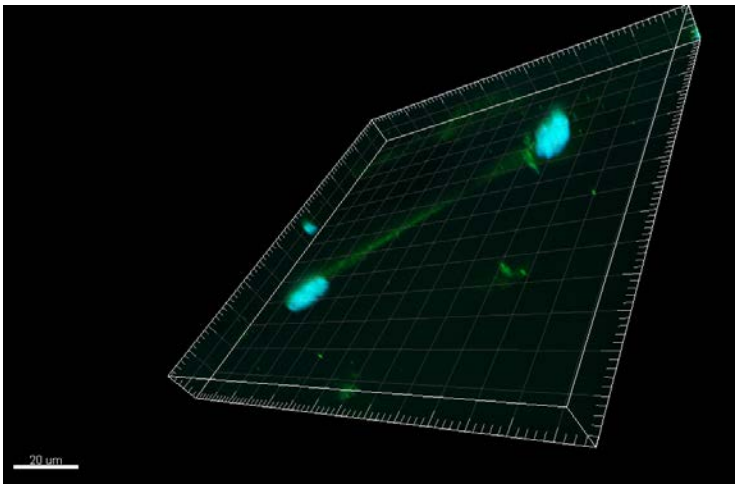


Figure 16. *Immunostaining of photoreceptor signature protein recoverine to evaluate the maturation of fRPCs. Recoverine = green, DAPI = blue*

However, the confocal reconstruction did not reveal the polarity of the cells in details. Unlike native environment where hierarchy is readily observed, the single cell culturing did not incorporate any situation for the cells to demonstrate polarity. This observation pinpointed that we need to introduce hierarchical environment such as competition or affiliation to demonstrate the polarity of cells during differentiation.

To maintain the stability of the fRPCs during differentiation, we modified the differentiation media by adding SU5402 and IWP2 to inhibit FGF and Wnt pathway. These modifications have been proven to have a positive effect during differentiation. With the adapted protocol, we observed photoreceptor maturation at day 14. As shown in **Fig. 17 (a)**, immunostaining of photoreceptor specific marker arrestin-3 indicated signature morphology of mature neurons. The expansion of the outer segment demonstrated development of rod photoreceptors. To further characterize the differentiation of fRPCs, we used western blot to examine the protein expression in the matrix. As shown in **Fig. 17 (b)**, the protein profile indicated abundance of both rhodopsin and M-opsin proteins after co-culturing for 14 days, which pinpointed formation of both rod and cone photoreceptors. Furthermore, we conducted real-time qPCR to verify the neuron formation. As shown in **Fig. 17 (c)**, the increase in presence of key neuron specific proteins such as Brn3, MATH5 and ISL-1 confirmed the maturation of photoreceptors using the bi-layer construct.

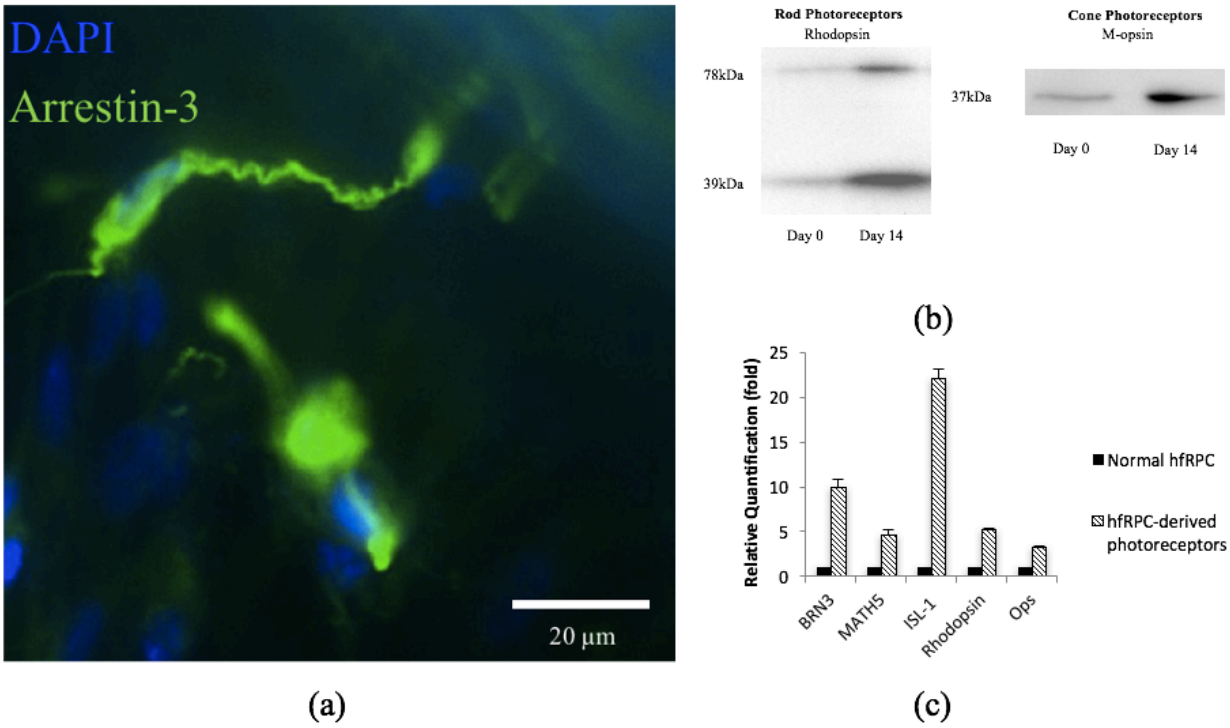


Figure 17 *(a) Immunostaining of cone photoreceptor specific protein arrestin-3 at day 14. (b) Western blot and (c) qPCR quantification of neuron-specific protein indicating photoreceptor maturation.*

As a conclusion, we have demonstrated bioprinting of multi-layer structure with cell-ECM matrix. Our immunostaining, as well as protein profiling results indicated that our co-culture system could recapitulate the native retinal development environment and enhance the maturation of photoreceptors.

What opportunities for training and professional development have the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
We have presented our research results in several invited talks in university seminars and professional meetings such as the Pre Retina Society Annual Meeting, Philadelphia, PA and Tissue Engineering and Regenerative Medicine International Society (TERMIS) annual meetings.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The layer-by-layer 3D bioprinting method will be a viable tool for retinal research. It could also be translational for future clinical uses in retina tissue regeneration. The hydrogel biomaterials

will also be useful for creating retinal tissue constructs that could be used for basic research such as retina disease modeling.

What was the impact on other disciplines?
The 3D bioprinting process is versatile in that it can be applied to other cell and tissue types. The 3D-printed retinal tissues could be used as in vitro models for early drug screening.

What was the impact on technology transfer?
We have created a startup company – Allegro 3D, Inc. in 2016.

What was the impact on society beyond science and technology?
Development of artificial retina tissues will change the clinical landscape by eliminating the current dependency on retina donor tissue and by providing a new strategy for restoring vision that would otherwise be lost in soldiers with severe retina blindness. The proposed 3D bioprinting and stem cell engineering represent an integration of emerging technologies that are truly novel for retina repair and regeneration.

5. Changes/Problems

Nothing to Report

6. Products

Journal Publications:

1. S.-H. Pyo , P. Wang , H. H. Hwang, J. Warner , W. Zhu , S.C. Chen, "Continuous Optical 3D Printing of Green Aliphatic Polyurethanes", *ACS Applied Materials & Interfaces*, December 20, 2016, DOI: 10.1021/acsami.6b12500
2. J. Warner, P. Soman, W. Zhu, M. Tom, and S.C. Chen, "Design and 3D Printing of Hydrogel Scaffolds with Fractal Geometries", *ACS Biomaterials Science*, Vol. 2 (10), pp 1763–1770, 2016.
3. J. Liu, H. H. Hwang, P. Wang, G. Whang, S.C. Chen, "Direct 3D Printing of Cell-laden Constructs in Microfluidic Architectures", *Lab on a Chip*, Vol. **16,** pp. 1430 – 1438, 2016. PMID: 26980159
4. W. Zhu, X. Ma, M. Gou, D. Mei, K. Zhang, S. C. Chen, "3D Printing of Functional Biomaterials for Tissue Engineering," *Current Opinion in Biotechnology*, Vol. 40, pp. 103–112, 2016. PMID: 27043763

Books or other non-periodical, one-time publications:

None

Other publications, conference papers, and presentations:

We have given the following seminars and presentation

- Corinne Bower Lecture, entitled "3D Bioprinting: Materials, Fabrication, and Tissue Engineering", in the Pre Retina Society Annual Meeting, Philadelphia, PA, 2014 (**Invited)**
- "Micro- and Nanoscale 3D Bioprinting for Functional Tissue Scaffolds" Functional Analysis and Screening technologies Congress, Boston, MA, Nov. 17-19, 2014. (**Invited)**
- "Micro and Nanoscale 3D Bioprinting: Materials, Fabrication, and Tissue Engineering," (Distinguished Seminar) University of California at Davis, March 12, 2015. (**Invited)**
- "Applications and Breakthroughs in Tissue Engineering and Bioprinting – What's Next?" in *Rock Stars of Innovation Summit*, San Diego, June 2015. (**Invited)**

- "Nano and Microscale 3D Bioprinting: An Enabling Technology for Personalized Regenerative Medicine", National Academy of Engineering (NAE) China-American Frontiers of Engineering Symposium, Irvine, CA, June 1-3, 2015. (**Invited)**
- "Genetics, Epigenetics, and Stem Cell Based Therapies for Blinding Eye Diseases", Dept. of Bioengineering, UC San Diego, May 1, 2015.
- "Micro and Nanoscale 3D Bioprinting for Functional Tissue Scaffolds", TERMIS 2015, Boston. (**Invited)**
- "Nano and Microscale Rapid 3D Printing for Functional Biomaterials", Mechanical Engineering Department, Univ of California at Berkeley, August 2015
- "Nano and Microscale Rapid 3D Printing for Regenerative Medicine", Yangming University, Taiwan, November 2015
- Keynote Talk: "Nano and Microscale Rapid 3D Printing for Regenerative Medicine", *5th ASME NanoEngineering for Medicine and Biology Conference (NEMB)*, Houston, Feb 22-23, 2016.
- "Rapid Scanningless 3D Printing: Vision, Status, and Research Needs," *NSF Additive Manufacturing for Health Workshop*, Arlington, VA, March 16-17, 2016.
- "Rapid 3D Bioprinting: an Enabling Technology for Precision Medicine", Bioengineering Department, Rice University, Feb 22, 2016.
- "Rapid 3D Bioprinting: an Enabling Technology for Creating Functional Tissue Models", Janssen Research & Development (Johnson and Johnson), March 2, 2016.
- "3D Bioprinting: An Enabling Technology for Tissue Engineering and Regenerative Medicine", *Nature Conference on Tissue Engineering and Regenerative Medicine* (Guangzhou, April 7–9, 2016).
- "3D Bioprinting: Interplay of Materials and Mechanics for Precision Tissue Engineering", Mechanical and Aerospace Engineering Department, UCSD, April 22, 2016.
- "Rapid 3D Bioprinting for Functional Scaffolds and Tissue Models", Rady Children's Hospital, May 20, 2016
- "3D Bioprinting for Functional in vitro Tissue Models", 2016 World Congress on In Vitro Biology, San Diego, June 2016 (**Invited**)
- "3D Bioprinting: Interplay of Materials and Mechanics for Precision Tissue Engineering", School of Mechanical and Automobile Engineering, Beijing Institute of Technology, June 27, 2016.
- "Rapid 3D Bioprinting for Precision Medicine", School of Medicine, Zhejiang University, July 11, 2016
- "Rapid 3D Bioprinting for Functional Scaffolds and Precision Tissue Models", Distinguished Seminar, Eli and Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research, University of Southern California, September 6, 2016.
- **"**3D Bioprinting for Functional in vitro Tissue Models", Center for Drug Discovery Innovation, UCSD, October 2016.
- "3D Printing of Functional Scaffolds and Precision Tissue Models", Department of Biomedical Engineering, University of Alabama, Birmingham, October, 2016
- "Rapid 3D Printing of Functional Scaffolds and Precision Tissue Models", Department of Mechanical Engineering and Materials Science, Duke University, October, 2016

- "Interplay of Materials and Mechanics in Rapid 3D Printing of Precision Tissues", Distinguished Seminar, Department of Medical Engineering, California Institute of Technology, November, 2016
- "3D BioPrinting: Technology Overview", Panelist for the workshop – "Perspectives on the Commercialization of Bioprinting in Regenerative Medicine". TERMIS-AM Annual Meeting, San Diego, December 11, 2016 (**Invited**)
- "Interplay of Materials and Mechanics in 3D Scaffolds through Rapid 3D Printing" Panelist for the workshop – "Development of New Biomaterial Scaffolds for Tissue Engineering". TERMIS-AM Annual Meeting, San Diego, December 11, 2016 (**Invited**)
- "Rapid 3D Bioprinting for Precision Tissue Engineering", Frontier Tech Forum San Diego, December 14, 2016 (**Invited**)
- "A 3D Printed Retinal Culture System for Photoreceptor Maturation" TERMIS 2016, San Diego.

Additionally, we and the Nature Journal Editors (*Nature Materials* and *Nature Materials Reviews* journals) organized a *Nature Conference on Tissue Engineering and Regenerative Medicine* in 2016.

Website(s) or other Internet site(s)
Dr. Chen's lab website updates news and publications: http://schen.ucsd.edu/lab/index.html
Dr. Chen's lab website updates news and publications: http://zhanglab.ucsd.edu

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Shaochen Chen
Project Role	PI
Researcher Identifier	NA
Nearest person month worked	1
Contribution to Project	Supervised the project, designed the experiments, and advised the graduate students
Funding Support	Partially from this grant.

Name	Kang Zhang
Project Role	Co-PI
Researcher Identifier	NA
Nearest person month worked	1
Contribution to Project	Co-supervised the project, co-designed the experiments, and

	co-advised the graduate students
Funding Support	Partially from this grant.

Name	Wei Zhu
Project Role	Graduate Student
Researcher Identifier	NA
Nearest person month worked	6
Contribution to Project	Carried out the experiments, and analyzed the results
Funding Support	Partially from this grant.

Name	Pengrui Wang
Project Role	Graduate Student
Researcher Identifier	NA
Nearest person month worked	10
Contribution to Project	Carried out the experiments, and analyzed the results
Funding Support	Partially from this grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. Special Reporting Requirements
See the Quad Chart in Appendices

9. Appendices

Selected Abstracts for presentation in university seminars and conferences
1) *Corinne Bower Lecture, Pre Retina Society Annual Meeting, Philadelphia, PA, 2014*

3D Bioprinting: Materials, Fabrication, and Tissue Engineering
Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center, Institute of Engineering in Medicine, University of California, San Diego

Abstract
The goal of our laboratory is to develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory's recent research efforts in femtosecond laser nano-printing and projection 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of micro-architecture, mechanical (e.g. stiffness and Poisson's ratio), chemical, and biological properties. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in

response to integrated physical and chemical stimuli. From these fundamental studies we can create both *in vitro* and *in vivo* tissue models for precision tissue engineering and regenerative medicine.

2) *(Distinguished Seminar) University of California at Davis, March 12, 2015*

3D Bioprinting: An Enabling Technology for Tissue Engineering and Regenerative Medicine

Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center
Institute of Engineering in Medicine
University of California, San Diego

Abstract

My laboratory aims to develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory's recent research efforts in 3D nano-printing and rapid 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of micro-architecture, mechanical (e.g. stiffness and Poisson's ratio), chemical, and biological properties. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in response to integrated physical and chemical stimuli. From these fundamental studies we can create both *in vitro* and *in vivo* tissue models for precision tissue engineering and regenerative medicine.

3) *National Academy of Engineering (NAE) China-American Frontiers of Engineering Symposium, Irvine, CA, June 1-3, 2015.*

Nano and Microscale 3D Bioprinting: An Enabling Technology for Personalized Regenerative Medicine

Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center, Institute of Engineering in Medicine, University of California, San Diego

Abstract

In my laboratory, we develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory's recent research efforts in femtosecond laser nano-printing and projection 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of physical, mechanical, chemical, and biological properties, aiming for personalized medicine. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in response to integrated physical and chemical stimuli. From these fundamental studies we can create both *in vitro* and *in vivo* tissue models for precision tissue engineering and regenerative medicine.

4) Tissue Engineering and Regenerative Medicine International Society (TERMIS) 2016 annual meeting, December 11-14, San Diego.

"**A 3D Printed Retinal Culture System for Photoreceptor Maturation**", Pengrui Wang, Wenqiu Wang, Wei Zhu, Kang Zhang, Shaochen Chen

The functional maturation of retinal photoreceptors, especially ganglion cells, from retinal stem cells (RSCs) are essential for personalized *in vitro* drug screening and potential *in vivo* retinal neuron repair. The expansion of ganglion cells is tightly related to the spatial arrangement of surrounding cell types such as the retinal pigment epithelium (RPE) and distribution of growth factors during embryonic eye development. While many studies have established systems to facilitate the maturation process, no study has demonstrated *in vitro* differentiation of ganglion cells from RSCs. The application of 3D printing technology to tissue engineering has enabled patterning of multiple cell types in a hierarchical biomimetic manner that can promote differentiation of RSCs. Here we present a 3D hydrogel-based co-culture model that isolated RSCs from RPE cells in a physiologically relevant 3D environment to mimic their original positioning during development stages. In comparison to 2D co-culturing system and single cell type model, our 3D construct show both phenotypic and developmental enhancements in the RSCs over weeks of culture. We used real-time, non-invasive quantitative phase imaging and immunochemistry staining to analyze the cell morphology changes. Our electrophysiological results also identified cell function by their distinctive discharge and membrane properties.

Layer-by-Layer Bioprinting of Stem Cells for Retinal Tissue Regeneration

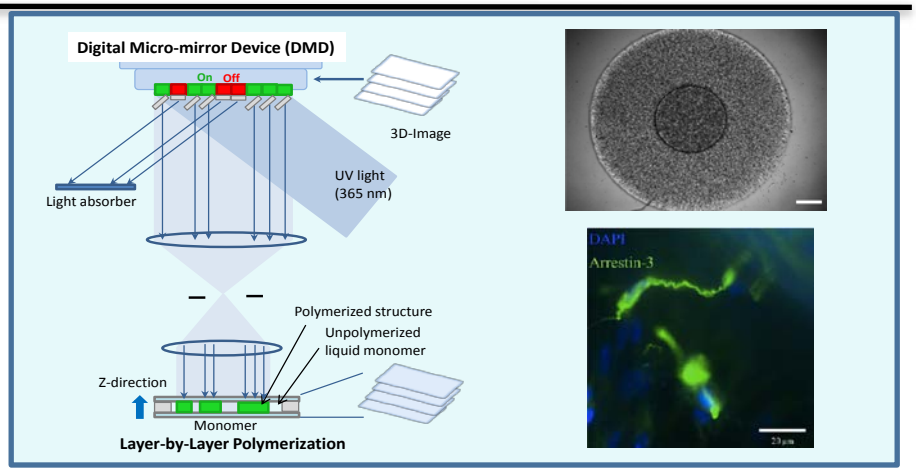
PI: Shaochen Chen and Kang Zhang **Org:** University of California, San Diego **Award Amount: $250,000**

Study/Product Aim(s)

- Aim 1: Develop and optimize a 3D bioprinting method with encapsulated retinal stem cells
- Aim 2: Layer-by-layer bioprinting of *in vitro* retina PRs /RPE/Bruch's membrane tissues

Approach

We hypothesize that through layer-by-layer bioprinting with retinal stem cells and appropriate growth factors (GF) encapsulated in a biomaterial (e.g. hyaluronic acid, HA), we can regenerate the anatomically correct retina in a biomimetic fashion, thus creating a paradigm shift in retinal tissue engineering.



(Left) A schematic of the 3D bioprinting system, (Top Right) Bioprinted core-shell structure (scale=500 μm), (Down Right) Immunostaining of cone photoreceptor specific protein arrestin-3

Timeline and Cost

Activities CY	14	15	16	17
Aim 1				
Aim 2				
Estimated Budget ($K)	**$125k**	**$125k**	**$000**	**$000**

Updated: 12/22/2016

Goals/Milestones

CY14 Goal –3D bioprinting development

- ☑ Synthesis of HA-GM
- ☑ Mechanical Testing of HA-GM
- ☑ 3D printing of HA-GM
- ☑ Create scaffolds with growth factors and retinal stem cells

CY15 Goals – LBL bioprinting of *in vitro* retina tissues

- ☑ Print HA scaffolds with retinal stem cells and different growth factors
- ☑ Characterize biological function of the 3D retinal tissue constructs